AN ANOMALOUS MENINGOCOCCUS.

By R. G. CANTI, M.B. (Cantab.).

Demonstrator of Pathology at St Bartholomew's Hospital.
Pathologist to the Alexandra Hospital, Queen Square, London.

(With Plates II.—IV.)

The meningococcus which forms the subject of this paper has already been alluded to in this *Journal* (1917, vol. xvi. p. 249). The outstanding features then reported were:

(1) That the original cultures showed minute colonies, a very few of which subsequently grew to resemble typical meningococcus colonies in size and appearance.

(2) That subcultivations from the minute colonies, throughout repeated generations, behaved precisely as the original cultures.

(3) That subcultivations from the large colonies, throughout repeated generations, produced only large colonies.

It is proposed to set forth in further detail the observations which were made and to describe certain experiments which were undertaken with a view to determining the nature of this peculiarity.

LITERATURE.

There is little to be found in literature bearing upon this subject, although variations in the size of meningococcus colonies have frequently been observed. Elser and Huntoon (1909) noted that certain strains produced colonies as small as 1 and $1\frac{1}{2}$ millimetres in diameter throughout repeated subcultivations and maintained that this peculiarity was only partly due to the medium employed.

Kohlisch (1915) obtained from the cerebro-spinal fluid a culture which showed three kinds of colonies, differing from one another in their cultural appearance and made up partly of Gram-positive and partly of Gram-negative cocci. Subcultivations from one of these colonies yielded the same three kinds of colonies together with a fourth kind. From the knee joint he isolated an organism having certain of the same peculiarities.

One of the kinds of colonies isolated, both from the cerebro-spinal fluid and from the knee joint, was minute. Emulsions from each source were strongly agglutinated by antimeningococcal serum.

HISTORY OF THE CASE.

The organism at present under discussion was isolated from the meninges of K. aged 18 years, a railway porter who was admitted to St Bartholomew's Hospital on May 26th, 1916, under the care of Dr Horton-Smith Hartley, to whom I am indebted for permission to publish this note. Four days previously he had fainted but returned to work the following day. He was admitted, having had a fit, and when examined was in a dazed condition and complained of headache. There was no rash and no other signs or symptoms were present. Two days later he became delirious and the neck muscles were stiff. Lumbar puncture was performed, purulent fluid withdrawn and the current Lister Institute serum injected intrathecally. This was repeated the following day. Delirium in the meanwhile had ceased but returned after two more days, accompanied by violence. The patient then lapsed into coma and died, having been ill nine days in all.

A necropsy revealed a slight exudate over the base of the brain. There was no exudate over the vertex. The convolutions were slightly flattened, but there was no obvious dilatation of the ventricles. The cord showed a purulent exudate which was most marked on the posterior aspect in the thoracic region. This exudate was much thicker than that at the base of the brain. The heart showed a few haemorrhages beneath the visceral layer of the pericardium. The intestines showed much congestion of the mucous membrane especially in the upper part of the jejunum. Elsewhere no abnormality was found. A culture of the heart's blood was sterile.

BACTERIOLOGY.

Examination of films of the deposit of the cerebro-spinal fluid collected ante-mortem showed an abundance of polymorphonuclear cells and large numbers of Gram-negative diplococci which had the morphological appearance of meningococci.

Cultures were made on blood ascites legumin agar from each of the only two specimens of fluid withdrawn. After 24 hours' incubation at 37° C. the plates were covered with minute translucent colonies which appeared like those of the influenza bacillus. They did not exceed 0·2 mm. in diameter and were scarcely visible to the naked eye. A hand

lens showed them to be colourless and slightly raised with a sharply defined circular outline. Under the low power of the microscope fine granulations were present over the whole of each colony. Films stained by the Gram-fuchsin method showed them to be composed of Gram-negative diplococci having the usual appearances of meningococci. From 24 hours onwards for the next five or six days a marked alteration occurred to about 1 in 200 of the colonies. Each successive day on which the culture was examined it was found that here and there a colony had apparently taken on a new lease of life and had rapidly grown to a large size. Such a colony attained a diameter of about 2 mm. in the 24 hours after the commencement of this sudden increase, and 3 or 4 mm. in the next two or three days, by which time it had the appearance of a normal meningococcus colony having a faint yellow pigmentation in its centre.

In the meantime the bulk of the colonies, though growing slightly, remained minute, never attaining a diameter of more than 0·5 mm. Certain of these small colonies however, which happened to be near to the large colonies, themselves began to grow larger between 24 and 48 hours after the large colonies had become developed. These ancillary or secondary large colonies attained diameters up to 1·5 mm., and those which were nearest to the primary large colonies were larger than those more remote.

Subcultivations of the small colonies always behaved in the same manner as the original cultures throughout repeated generations for seven months, during which time the organism was carried through the single colony stage on more than 20 occasions. At the end of this time however the characteristics of the strain propagated became somewhat modified and the differentiation between the large and small colonies less conspicuous. The primary large colonies still appeared, but the secondary large colonies were rarely or poorly formed and the small colonies grew to a somewhat larger size and were less translucent.

Subcultivations from primary large colonies were always found to grow nothing but large colonies in 24 hours, and henceforward subsequent generations behaved like the typical meningococcus.

The same characteristics were found when small colonies were sown on to inspissated egg medium. After a week's incubation no more large colonies appeared but cultures were found to be alive after a month or more. By subcultivation from a mixture of small colonies on to legumin agar plates and then picking out a single small colony, the strain was kept going from time to time.

Ascites broth cultures of single small colonies 24 hours old, sub-cultivated on to legumin agar showed a pure growth of small colonies after 24 hours' incubation and subsequently behaved in the same way as cultures made direct from a single small colony.

The strain was submitted to Dr A. Eastwood who kindly made cultivations and found that the growth in Kutscher's agar had the same appearance as on legumin agar.

In the course of these investigations many batches of legumin agar were employed, and a large number of other meningococci cultivated on them showed, on all occasions, a vigorous and typical growth.

It might be argued that the nature of the large growing colonies was dependent upon the heterogeneous constitution of the medium, a large colony appearing at a spot where the medium possessed a quality particularly beneficial for its growth. But in view of the fact that the proportion of large to small colonies was the same on the various media employed, and on various batches of them, such a possibility can be excluded. Further this theory would not explain why the large colonies cropped up at more or less regular periods, for it would be expected that all the large colonies would appear at the same time.

ANALYTICAL OBSERVATIONS.

The table shows certain observations of the number of large colonies present on the plates after various periods of incubation. The first two columns show the figures obtained from the two original cultures of the cerebro-spinal fluid, and the remainder those obtained from sub-cultivations of the small colony line inoculated at various dates. The figures indicate the count of large colonies from day to day. Those plates on which the greatest number of large colonies appeared were observed to be the most heavily inoculated.

TABLE.

	May	May	May	June	July	August	August
24 hours' incubation	0	3	0	0	0	0	0
48 „ „		36	19	9	4	17	9
72 „ „		55	34	?	17		
96 „ „			79	41	23		
120 „ „			81		?		
144 „ „					24		

A count was made on one occasion of both the large and small colonies and their relative numbers found to be 4700 and 24 respectively, that is approximately 200 to 1. During the months over which these

observations were carried on no noticeable alteration in proportion occurred.

It is seen that the large colonies increase in numbers more or less uniformly from day to day after the first 24 hours till about the fourth day after which time the appearance of a new large colony is rare.

Plates II—IV, Figs. 1—5, show successive photographs of a culture from a single small colony which had previously passed many times through the single colony stage. They were taken after 1, 2, 3, 4 and 6 days' incubation respectively, and are enlarged 1·75 diameters.

It is seen that in Fig. 1 the colonies are minute and that no large colonies are apparent[1].

In Fig. 2 primary large colonies are beginning to show themselves, and in Fig. 3 these have grown to a considerable size and more such have appeared. That these primary large colonies are due to the increase in size of small colonies and not to the appearance of new colonies is shown by selecting certain of the large colonies which happen to be in a thinly populated portion of the culture and tracing them back to earlier photographs where they are seen as small colonies identical with their fellows.

Fig. 4 shows the large colonies to have increased greatly in size and to exhibit a slight darkening in their centre. This is due to the faint yellow pigmentation to which reference has already been made. It is more apparent in the photographs than it was on the actual culture as the photographic plate employed was not "isochromatic." Around those primary large colonies which had existed as such for more than 24 hours, the groups of secondary large colonies are seen. Their gradual increase in size according to their situation is well demonstrated in those instances where they happen to lie in a straight line leading up to a primary large colony.

Fig. 5 shows the same changes in a more advanced stage. Incubation subsequent to this time showed no further increase in growth although the plates were sealed with paraffin wax to prevent evaporation.

Measurements have been made on the photographs with a view to ascertaining whether the centre of a newly formed large colony corresponds with the centre of the small colony from which it arose. It was found that whereas in some cases the two appear to be concentric yet

[1] These photographs are entirely untouched, and certain marks which might at first sight be mistaken for colonies are due to (1) flaws in the photograph which do not appear subsequently or (2) flaws in the agar medium which may be traced unaltered throughout the series.

in others the two centres undoubtedly do not coincide. This observation suggests that the sudden enlargement of the colony is not of a uniform nature, but it is due to a localized change commencing in some part of the colony, and possibly in one individual, a papilla being formed which rapidly overgrows the small colony and itself becomes paramount. If this is so the large colonies would appear to be analogous to the dulcite fermenting papillae of *B. typhosus* and other similar papillae recently studied by Penfold (1911—1912—1913) with the difference that instead of many papillae appearing on one colony only one papilla was formed, and that on only a few colonies.

AGGLUTINATION.

Agglutination tests were kindly carried out by Professor Andrewes on two emulsions each prepared from cultures of single small colonies and on two emulsions each prepared from single large colonies. All four emulsions were agglutinated to approximately the same titre by Gordon Type II serum (Gliddon) and not with Type I or Type III sera.

A rabbit serum was prepared from an emulsion of small colonies and was found to agglutinate the homologous coccus and other cocci of Type II to a high titre.

CARBOHYDRATE REACTIONS.

Glucose ascites litmus broth tubes sown with organisms from each kind of colony were fermented in three days. Similar cultivations in saccharose were not fermented in one week.

ADJUVANT BROTH EXPERIMENTS.

The observation that those small colonies in close proximity to the large colonies themselves increased in size suggested the probability that the medium surrounding the primary large colonies became modified by them in a manner beneficial to the small colonies. Accordingly the following experiment was carried out.

A primary large colony was selected from a young culture of a line of small colonies which had passed through the single colony stage three times since isolation. It was inoculated into an Erlenmeyer flask containing about 200 c.c. of broth mixed with fresh sterile ascites fluid. A similar flask of uninoculated ascites broth was also prepared as a control. Both flasks were incubated for 23 days at 37° C. The inoculated broth (which on cultivation gave a pure growth of meningococci) was

then filtered through a Berkefeld filter under aseptic conditions and the filtrate incubated for a few days to prove its sterility. Two "stab" tubes each containing 16 c.c. of legumin agar were then melted and cooled to 45° C. To one of them was added 4 c.c. of the filtrate and to the other 4 c.c. of the control broth. Plates were then poured from these mixtures and each was inoculated with a loopful of a 24 hour ascites broth culture from a single small colony of a line which had passed through the single colony stage on twelve previous occasions. The plate containing the filtrate showed after 24 hours' incubation a copious growth of normal sized colonies, among which there were many small colonies in the more crowded areas. After 48 hours' incubation there was a general increase in the size of all the colonies although a fair number of rather small colonies still remained. On the control plate the culture showed the usual characteristics of the strain. There was a profuse growth of minute colonies at the end of 24 hours and after a further 24 hours nine colonies had attained the size of a normal 24 hour old meningococcus colony.

The experiment was repeated and a similar and even more striking difference was observed between the filtrate culture and the control.

Plate IV, Figs. 6 and 7, shows photographs, enlarged to 1·75 diameters, of the two cultures of the first experiment taken after 48 hours' incubation.

It is thus seen that legumin agar containing 20 % of the filtrate of a broth culture prepared from a large colony markedly increases the growth of colonies of the small growing line.

DISCUSSION.

The large colonies which are formed appear to be identical with typical meningococcus colonies. They have been observed to spring from an atypical strain in which the colonies formed were minute. In this respect the strain is a rarity.

According to De Vries (1906) evolution takes place by three kinds of steps, progressive, retrogressive, and degressive. The first of these steps is achieved by the acquirement of a new character, the second by the loss or latency of a character, and the third by the reappearance of such a latent character. Now if the forward or positive step produces a variety which is identical with a variety commonly found and further produces it from an uncommon variety, it seems permissible to assume that the newly formed variety arose by the reappearance of a character latent in its ancestors and is therefore degressive. In this case the coccus

producing the small colonies would be of a retrograde or negative variety having arisen by the latency or loss of a character which reappears in certain of its progeny by a degressive step, the common variety being again evolved.

With regard to the nature of the latent character which reappears, the adjuvant broth experiments show that the large colonies produce a substance which when mixed with the nutrient medium will cause a culture of the small colony strain to develop much larger colonies than on the nutrient medium alone. It would therefore appear that the character latent in the cocci which produces small colonies is the capacity to produce a substance beneficial for their growth. The existence of such a substance has been suggested in connection with bacterial "lag" as the possible reason why maximal growth does not occur in broth cultures of *Bacillus coli* till some time after incubation has been commenced: the idea, however, is not consistent with all observations recorded. (Penfold, 1914.)

The ancillary relation of one kind of organism to another where the former grows with greater vigour in the neighbourhood of the latter is a well recognised fact, and in nature generally it is frequently observed that two individuals of different kinds may live in proximity for their mutual benefit. Further it is generally supposed that individuals produce substances which are always useless and frequently inimical to their own growth and that of their kind. The above experiments, however, seem to indicate that in at least some cases a living individual produces a substance which is necessary for its well being, an observation which is totally contrary to general experience. What the nature of this substance may be is a matter of conjecture. Consideration must be given to the possibility of the formation of acid by the large colonies till a hydrogen ion concentration is attained which is optimum for growth. That the substance in question belongs to the "vitamine" class seems improbable since the original cultures were made in a medium containing fresh blood in which presumably the necessary "vitamines" were present in sufficient quantity. It however seems admissible to assume that the organisms in the large colonies may be capable of producing a ferment or of otherwise breaking down the too highly complex nutritive material in the medium, and may thus prepare for themselves a substance which they are capable of assimilating. In this case the adjuvant broth would contain this substance which has been formed during its incubation in excess of the requirements of its population, and would then be able to supply nutritive material in the required form to those cocci which

Fig. 1.

Fig. 2.

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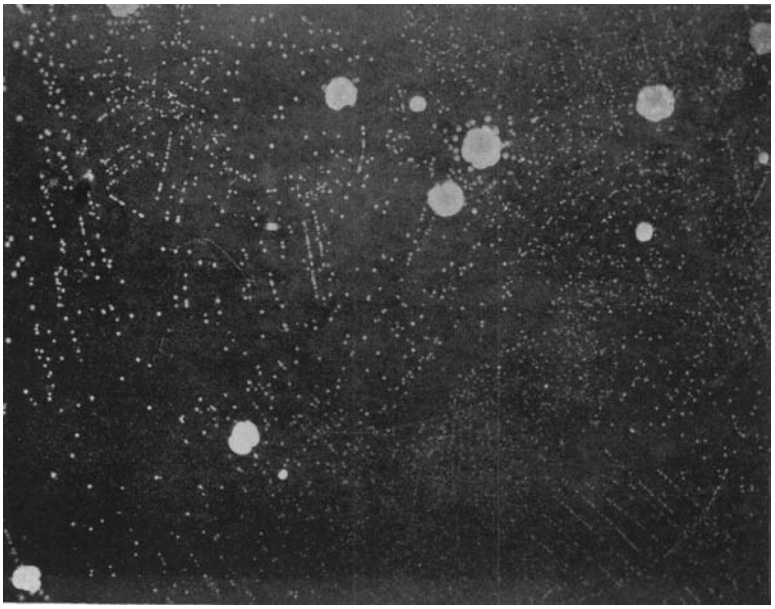

Fig. 3.

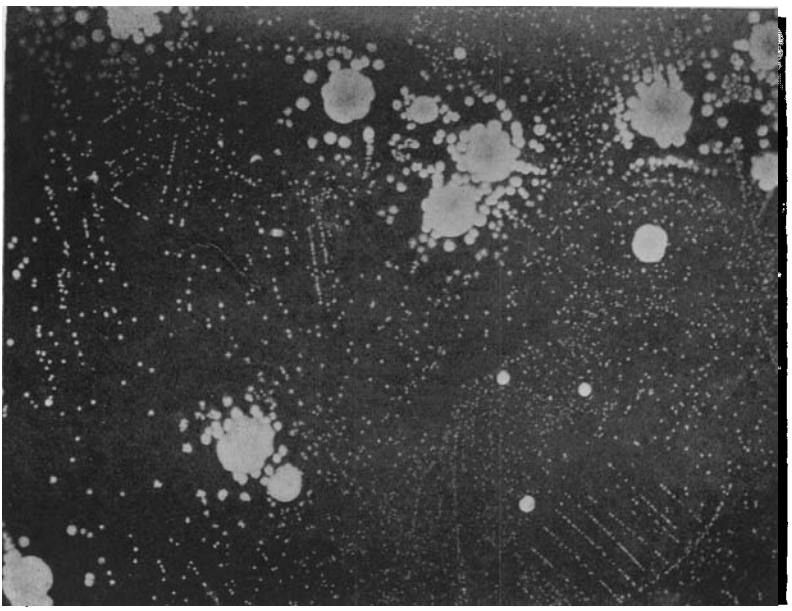

Fig. 4.

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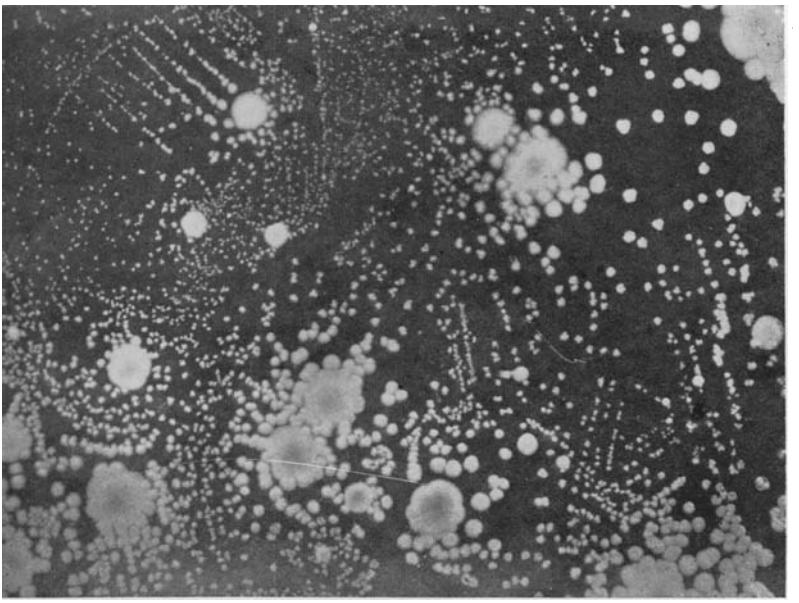

Fig. 5.

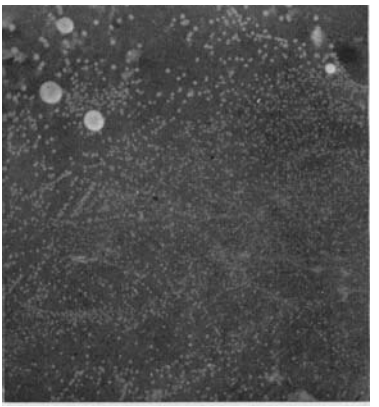

Fig. 6.

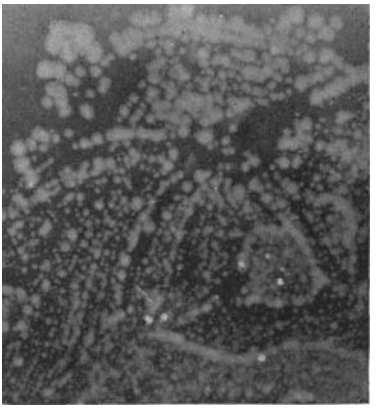

Fig. 7.

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lacked the capacity of converting the more highly complex substance to their use.

SUMMARY.

(1) An unusual strain of meningococcus has been isolated which produces minute "dew drop" colonies. This character has persisted throughout a large number of successive generations. Every culture, however, has shown lateral off-shoots consisting of colonies which have the size and appearance of those of typical meningococci. In subsequent subcultivations from these colonies the newly acquired size and appearance remain constant.

(2) It is suggested that the strain isolated is a retrograde variety and that the off-shoots are produced by degressive evolution.

(3) It is also suggested that the character which reappears in the degressive step is that of producing a substance beneficial for growth.

It is impossible, however, to offer any satisfactory explanation of the phenomena observed without a more extended series of experiments. This was impracticable at the time owing to pressure of routine work and further observations on the same organism have now become impossible owing to the fact that the small growing strain has died out. Nevertheless the findings seem worth recording in their present state in the hope that they may be of use to others who have met or may meet with similar peculiarities in the course of their work.

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